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S. WALSH

G. Bissonnette

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Survival of Chlorine-Injured Enterotoxigenic *Escherichia coli* in an In Vitro Water System†

SUZANNE M. WALSH‡ AND GARY K. BISSONNETTE*

Division of Plant and Soil Sciences, P.O. Box 6057, West Virginia University, Morgantown, West Virginia 26506-6057

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Survival of chlorine-injured and noninjured subpopulations of enterotoxigenic *Escherichia coli* was compared in KH_2PO_4-buffered water and chlorine-neutralized tap water. Injured cells were no less persistent than noninjured cells and did not exhibit limited survival as a consequence of chlorine injury. At high inoculum densities, some injured cells were able to repair, apparently owing to the accumulation of materials arising from the chlorination procedure.

Investigations concerning the health risk associated with sublethally injured indicator organisms in chlorinated environments have recently focused on the effect of injury on pathogen virulence. Most studies indicate reduced virulence as a consequence of chlorine injury (3, 5, 6). This is, however, not a permanent condition since chlorine-injured enteropathogens regain lost virulence factors after repair (3, 6). Singh et al. (4) demonstrated that chlorine-injured enterotoxigenic *Escherichia coli* (ETEC) was able to repair and resume heat-stable enterotoxin production in mouse ligated ileal loops. Their findings raise questions concerning the potential for in vivo repair and the subsequent pathogenicity after ingestion of sublethally injured enteropathogens. Little information, however, is available regarding the in situ fate of injured enteropathogens. Thus, it was the objective of our study to examine the survival characteristics of chlorine-injured ETEC in an aquatic system under laboratory conditions.

ETEC H10407 (O28:H11), the strain used in our previous study to evaluate the effect of chlorine injury on heat-labile enterotoxin production (6), was provided by R. Wilson from Pennsylvania State University, University Park, Pa. The test organism was grown in Trypticase soy broth at 37°C, 120 rpm, for 18 h and stored at 5°C for use as a stock culture. All media used from BBL Microbiological Systems, Cockeysville, Md. The organism was grown under identical conditions for survival experiments. For each experiment, 18-h cultures were harvested by centrifugation at 3,020 × *g*, washed three times, and suspended in 6.25×10^{-4} M KH_2PO_4-buffered water (pH 6.7) which had previously been rendered chlorine-demand-free (1). Inoculum (20 ml) was then added to a 1-liter flask containing 480 ml of the buffered water and stirred at a constant slow speed. Flasks prepared in this manner typically contained 1.0×10^8 to 1.5×10^8 CFU/ml. Cells were injured by exposure to approximately 0.55 mg of chlorine per liter prepared from a fresh stock solution of 5.25% sodium hypochlorite (commercial bleach; Clorox). Exact chlorine measurements were not made because our purpose was to induce ≥90% injury to the test population, rather than identify a specific chlorine level for injury. The reaction was allowed to proceed for 6 to 7 min and then was terminated by the addition of 10 ml of 20% sodium thiosulfate. Survivors were enumerated by the spread plate technique immediately upon neutralization (0 h) and at selected times thereafter. Injury was assessed by comparing triplicate plate counts after 18 h of incubation at 35°C on tryptone glucose yeast extract (TGE) agar and M-fecal coliform (M-FC) agar (M-FC broth containing 15 g of agar per liter). Only populations of *E. coli* incurring ≥90% injury after chlorine exposure and neutralization (at least 10-fold fewer survivors were detected on selective M-FC agar, compared with nonselective TGE agar) were used in subsequent persistence studies.

Populations of ETEC incurring ≥90% injury were monitored in the reaction flask for a period of 72 h after neutralization (Fig. 1). Of particular interest was a portion of the injured population that was able to repair in this system. During the first 12 h after neutralization, the number of survivors recovered on M-FC agar increased 89%. This was accompanied by a 30% increase on TGE agar. Less than a third of the increase on M-FC agar, therefore, was attributable to multiplication within the noninjured subpopulation. The remainder was caused by repair of injured cells. Multiplication was evident in this system after 12 h. Similar increases were detected between 12 and 72 h by both media. Apparently, materials that accumulate in the chlorination system as a result of the procedure permit multiplication. In comparison, non-chlorine-exposed ETEC showed a slight decrease in the number of survivors on both TGE and M-FC media during the 72-h period.

In separate experiments, samples were removed from the chlorinated system immediately after neutralization and subsequently were reinoculated into 500 ml of sterile KH_2PO_4-buffered water (Fig. 2) or sterile chlorine-neutralized tap water (Fig. 3). Die-off was apparent at these lower population densities, but there was no evidence of increased susceptibility to continued exposure to buffered or chlorine-neutralized tap water among injured ETEC. Reinoculation of chlorinated ETEC into sterile buffer resulted in an 80% decrease in the total population and a 76% decrease in the number of survivors on M-FC agar after 72 h (Fig. 2). Exposure of chlorinated cells to chlorine-neutralized tap water for 8 h resulted in an 80% decrease in the number of survivors as determined on both media (Fig. 3). The largest decline in the number of recoverable cells occurred within the first hour, as reflected by 55 and 64% fewer cells detected by TGE and M-FC agar, respectively. Statistical analyses

* Corresponding author.

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‡ Present address: Department of Biology, Westfield State College, Westfield, MA 01086.

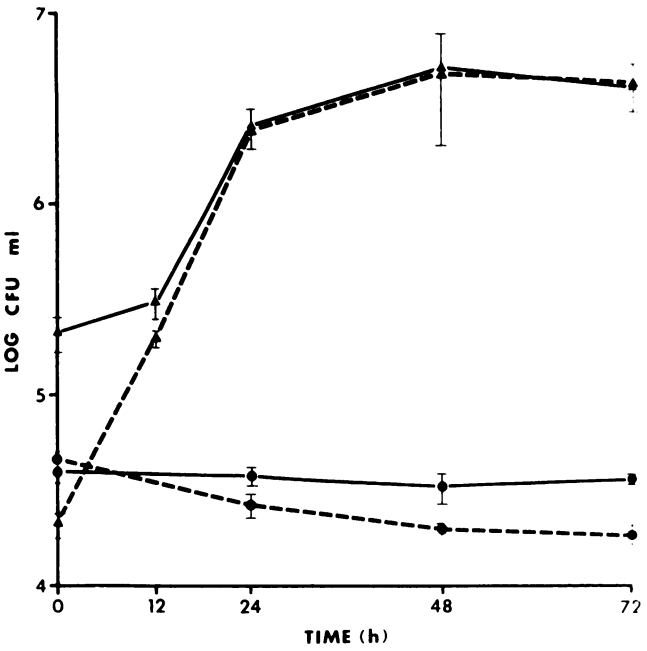

FIG. 1. Persistence of ETEC in exposure systems. Comparison of nonselective TGE (——) and selective M-FC (-----) counts after chlorination and neutralization (▲) and buffered-water exposure (●). Data was gathered from six observations (two independent experiments with triplicate platings). Bars represent ±1 standard deviation.

(analyses of variance of logarithmically transformed counts) gave no evidence of significant differences ($P > 0.05$) in the slopes of persistence curves generated by colony counts obtained on nonselective and selective media (Fig. 2 and 3).

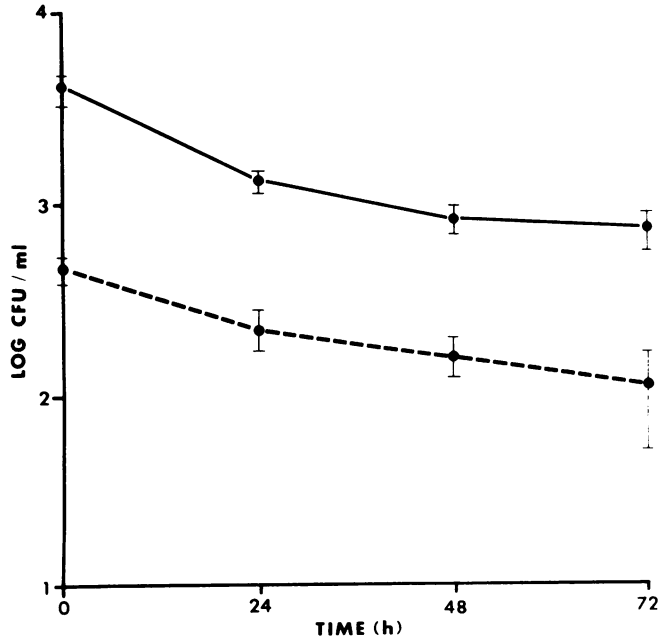

FIG. 2. Persistence of chlorinated ETEC after reinoculation into sterile buffered water. Comparison of nonselective TGE (——) and selective M-FC (-----) counts. Data was gathered from nine observations (three independent experiments with triplicate platings). Bars represent ±1 standard deviation.

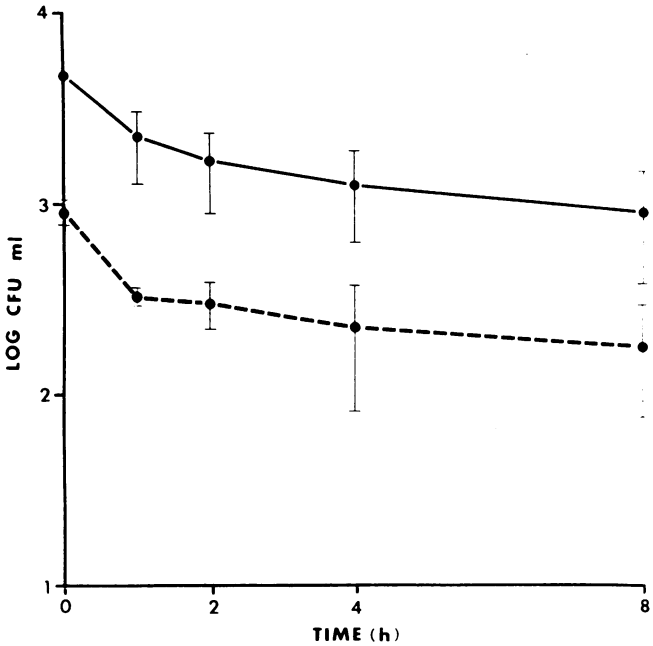

FIG. 3. Persistence of chlorinated ETEC after reinoculation into sterile neutralized tap water. Comparison of nonselective TGE (——) and selective M-FC (-----) counts. Data was gathered from nine observations (three independent experiments with triplicate platings). Bars represent ±1 standard deviation.

The reinoculation of samples from the chlorinated system into KH_2PO_4-buffered and chlorine-neutralized tap water resulted in an approximately 500-fold dilution of killed cells and dead cell debris. Under these conditions, no multiplication or repair was demonstrated (Fig. 2 and 3). Since failure to transport glucose and protein during sublethal chlorine injury has been demonstrated (2, 6), the role of exogenous nutrients in the repair process is unclear. Our results show that repair is possible in low-nutrient, aquatic environments which support minimal allochthonous growth (Fig. 1). These findings raise new questions concerning the role of nutrients in initiation of the repair process and the health risks associated with chlorine-injured pathogens in aquatic environments. In addition, it may be of some significance that multiplication of enteropathogens can occur when supported by materials released from damaged cells after the chlorination procedure. Our experiments further demonstrate that injured cells can persist as such in the environment and exhibit survival characteristics similar to noninjured cells. In view of these findings and the potential for in vivo repair (4), the use of extraordinary methodologies directed towards recovery of injured indicator organisms from chlorinated environments appears to be justified.

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LITERATURE CITED

1. **American Public Health Association.** 1985. Standard methods for the examination of water and wastewater, 16th ed. American Public Health Association, Washington, D.C.
2. **Camper, A. K., and G. A. McFeters.** 1979. Chlorine injury and the enumeration of water-borne coliform bacteria. Appl. Envi-

ron. Microbiol. **37**:633–641.
3. **LeChevallier, M. W., A. J. Singh, D. A. Schiemann, and G. A. McFeters.** 1985. Changes in virulence of waterborne pathogens with chlorine injury. Appl. Environ. Microbiol. **50**:412–419.
4. **Singh, A., R. Yeager, and G. A. McFeters.** 1986. Assessment of in vivo revival, growth, and pathogenicity of *Escherichia coli* strains after copper and chlorine-induced injury. Appl. Environ. Microbiol. **52**:832–837.
5. **Walsh, S. M., and G. K. Bissonnette.** 1983. Chlorine-induced damage to surface adhesins during sublethal injury to enterotoxigenic *Escherichia coli*. Appl. Environ. Microbiol. **45**:1060–1065.
6. **Walsh, S. M., and G. K. Bissonnette.** 1987. Effect of chlorine injury on heat-labile enterotoxin production in enterotoxigenic *Escherichia coli*. Can. J. Microbiol. **33**:1091–1096.